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Letter

Crystal structure of thrombin in complex with fibrinogen γ′ peptide

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Abstract

Elevated levels of heterodimeric γ_A/γ' fibrinogen 2 have been associated with an increased incidence of coronary artery disease, whereas a lowered content of γ′ chains is associated with an increased risk of venous thrombosis. Both situations may be related to the unique features of thrombin binding to variant γ′ chains. The γ′ peptide is an anionic fragment that binds thrombin with high affinity without interfering directly with substrate binding. Here we report the crystal structure of thrombin bound to the γ′ peptide, solved at 2.4 Å resolution. The complex reveals extensive interactions between thrombin and the γ′ peptide mediated by electrostatic contacts with residues of exosite II and hydrophobic interactions with a pocket in close proximity to the Na^+ binding site. In its binding mode, the γ′ peptide completely overlaps with heparin bound to exosite II. These findings are consistent with functional data and broaden our understanding of how thrombin interacts with fibrinogen at the molecular level.

Keywords: Thrombin; Fibrinogen; γ′ peptide; Serine protease structure; Allostery

Fibrinogen is a dimeric protein whose subunits are composed of three polypeptide chains named Aα, Bβ and γ [1]. Two forms of fibrinogen, fibrinogen 1 and fibrinogen 2, differ with respect to the C-terminal of their γ chains and can be separated by ion exchange chromatography [2,3]. Fibrinogen 1 contains a homodimeric γ polypeptide (γ_A/γ_A), each composed of 411 residues. Plasma contains approximately 15% fibrinogen 2 that exists as a heterodimer (γ_A/γ'), having a variant γ′ chain which originates from an alternative mRNA splicing between exons 9 and 10 [4]. The variant γ′ chain has 427 residues, with a highly anionic C-terminal segment, V^{408}RPEHPAETEY$_S$DSLY$_S$PEDDL^{427}, carrying seven Glu and Asp residues and two sulfonated Tyr (Y_S) residues at positions 418 and 422. Upon binding of the E domain of fibrin(ogen) to thrombin via exosite I [5,6], fibrinopeptides A and B are cleaved off the Aα and Bβ chains promoting the polymerization of fibrin into an insoluble clot. However, thrombin binds fibrinogen with high affinity at an additional site provided by the γ′ chains of fibrinogen 2 [7]. The clinical relevance of the γ′ chain variant of fibrinogen is that clots formed from fibrinogen 2 are less susceptible to lysis [8,9], and elevated levels of the heterodimer γ_A/γ' have been associated with an increased incidence of coronary artery disease [10]. In addition, a lowered content of γ′ chains is associated with an increased risk of venous thrombosis [11]. Both situations may be related to the unique features of thrombin binding to variant γ′ chains.

Previous studies have suggested that the γ′ peptide fragment ^{414}AETEY$_S$DSLY$_S$PEDDL^{427} binds to thrombin via exosite II [12], and that replacement of Y_S with Phe residues at position 418 and 422 abolishes thrombin binding [7]. Here we present the 2.4 Å crystal structure of thrombin complexed with the γ′ fragment V^{408}RPEHPAETEY$_P$DSLY$_P$PEDDL^{427}, where the Y_S residues at position 418 and 422 have been replaced with phosphorylated Tyr (Tyr_P or Y_P) residues. The replacement is inconsequential on thrombin binding [9,12]. The structure (Table 1) shows that the γ′ peptide interacts with exosite II of thrombin (Fig. 1) in a way that reproduces closely the binding of heparin [13], in agreement with recent functional results

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Table 1
Crystallographic data of thrombin in complex with fibrinogen γ′ peptide

Data collection:	PDB ID code 2HWL
Wavelength (Å)	0.9
Space group	$P2_1$
Unit cell dimensions (Å)	a=66.40
	b=81.52
	c=70.26
Resolution range (Å)	40.0–2.4
Observations	140227
Unique observations	27503
Completeness	98.8 (94.7)
R_{sym} (%)	6.5 (30.8)
I/σ(I)	20.0 (2.8)
Refinement:	
Resolution (Å)	40.0–2.4
$\|F\|/\sigma(\|F\|)$	>0
R_{cryst}, R_{free}	0.214, 0.249
Reflections (working/test)	25293/1323
Protein atoms	4705
Solvent molecules/Na^+	133/2
Rmsd bond lengths[a] (Å)	0.007
Rmsd angles[a] (°)	1.6
Rmsd B values (Å^2) (m.c./s.c.)[b]	1.78/2.63
<B> protein (Å^2)	47.6
<B> Na^+ (Å^2)	45.9
<B> solvent (Å^2)	43.6
Ramachandran plot[c]:	
Most favored (%)	98.1
Generously allowed (%)	1.2
Disallowed (%)	0.6

[a] Root-mean-squared deviation (Rmsd) from ideal bond lengths and angles and Rmsd in B-factors of bonded atoms.
[b] m.c., main chain; s.c., side chain.
[c] Calculated using PROCHECK [21].

[9,12,14,15]. Exosite II is composed mainly of cationic residues and Arg-93, Arg-101, Arg-233, Lys-236 and Lys-240 offer electrostatic complementarity to the anionic moiety of the γ′ peptide. Tyr_P-418 interacts with residues Arg-126, Lys-235 and Lys-236, while Tyr_P-422 interacts with Lys-240 (Table 2, Fig. 1). Pro-413 and Ala-414 of the γ′-peptide penetrate a hydrophobic pocket of thrombin formed by residues Ile-162, Val-163, Arg-165, Phe-181, Ala-183 and the disulfide bond Cys-168: Cys-182, which are in close proximity to the side chain of Tyr-225 near the Na^+ binding site. Interaction of the γ′ peptide with this pocket could trigger inhibitory allosteric effects on the active site and explain the delayed release of fibrinopeptide A, the delayed generation of α-profibrin, and the slower cleavage rate of the factor XIII activation peptide [9].

Tyr_P-418 wedges its negatively charged moiety in a positively charged groove formed by the side chains of thrombin Arg-126, Lys-235, and Lys-236 (Fig. 1). The O1, O2 and O3 atoms of the phosphate group are in close contact with the NH1 group of Arg-126 and the Nζ atoms of Lys-235 and Lys-236 (Table 2). This strong electrostatic interaction explains most of the high affinity of the γ′-peptide for thrombin. The phosphate group of Tyr_P-422 makes electrostatic interactions with Lys-240 (Table 2, Fig. 1) and also contacts Arg-126 of a symmetry-related molecule. Furthermore, Arg-93, Lys-235, and Lys-236 of a symmetry-related molecule engage the C-terminal portion of the γ′ peptide in electrostatic interactions (Table 2). The ability of the γ′ peptide to bridge two thrombin molecules in the crystal lattice may be revealing of the mechanism through which the fibrin clot sequesters thrombin in solution.

It is of interest to compare our structural data with the recent NMR investigation of the γ′ peptide bound to thrombin reported by Sabo et al. [16]. Based on the NMR data, the γ′ peptide segment 412–427 interacts with exosite II mostly through Tyr_P-422 [16]. Our structure shows Tyr_P-422 interacting with Lys-240, but also with Arg-126 in exosite II of a

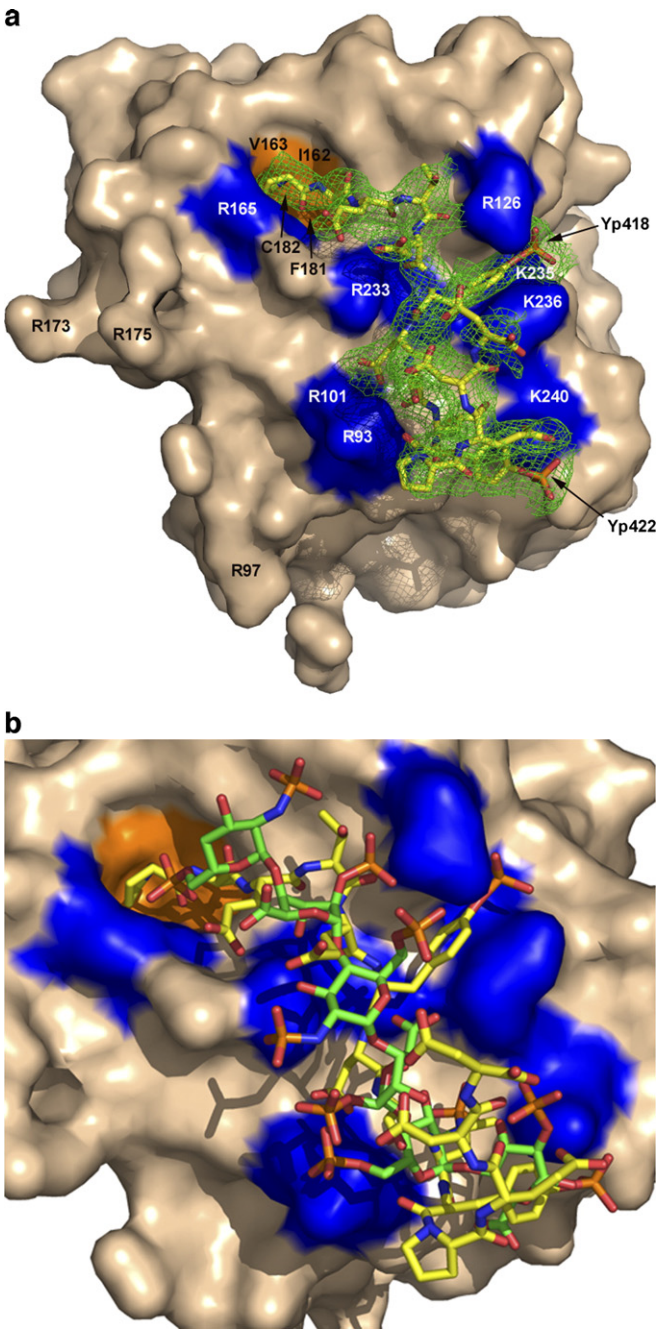

Table 2
Thrombin residues involved in recognition of γ′ peptide

Exosite II residues	Distance[a] (Å)	γ′ peptide residues
Molecule 2 in the asymmetric unit		
Val-163 O	3.12	Pro-413 N
Arg-165 NH_2	2.97	Pro-413 O
Arg-233 NH_2	3.03	Glu-415 Oε1
His-230 Nε2	3.27	Glu-415 O
Arg-126 NH_2	2.47	Thr-416 O
Arg-126 NH_1	2.56	Tyr_P-418 O1
Arg-126 Nε	3.00	Tyr_P-418 O1
Lys-235 Nζ	2.94	Tyr_P-418 O2
Lys-236 Nζ	2.91	Tyr_P-418 O3
Arg-101 Nε	2.59	Asp-419 Oδ1
Asn-179 Nδ2	3.02	Asp-419 Oδ2
Arg-233 O	3.02	Asp-419 N
Arg-101 NH_2	3.24	Asp-419 Oδ2
Arg-93 NH_1	2.54	Ser-420 O
Lys-240 Nζ	2.79	Tyr_P-422 O2
Symmetry-related molecule		
Arg-126 NH_1	3.65	Tyr_P-422 OH
Arg-126 NH_2	3.70	Pro-423 O
Lys-235 Nζ	3.76	Glu-424 Oε2
Arg-93 NH_2	3.58	Asp-425 Oδ1
Arg-93 NH_2	2.51	Asp-426 Oδ1
Arg-93 NH_2	3.99	Asp-426 Oδ2

[a] Only contacts<4 Å are reported.

symmetry-related molecule (Table 2, Fig. 1). Interestingly, Sabo et al. [16] identified a structural feature of the γ′ peptide bound to thrombin, i.e., a β-turn involving residues from Tyr-422 to Asp-425. The turn is also evident in our X-ray structure and causes the C-terminal of the γ′ peptide bound to thrombin to bridge exosite II of a symmetry-related molecule (Fig. 1). The study also suggested an involvement of residues Arg-93, Arg-97, Arg-173, and Arg-175 in the recognition of the γ′ peptide [16]. Except for Arg-93, the X-ray structure does not show any interaction of the γ′ peptide with these residues (Fig. 1). However, our crystal structure was refined to 2.4 Å resolution and contains electron density only for residues 413–426 of the γ′ peptide. This leaves the exact position of residues 408–412 at the N-terminal and Leu-427 at the C-terminal unknown. The N-terminal residues 408–412 modeled in an extended conformation fitted to weak electron density could reach the opposite end of exosite II in the vicinity of Arg-173 and Arg-175. However, Arg-97 is too distant from the bound γ′ peptide and its importance is not confirmed by the crystal structure (Fig. 1).

Our structure reveals the molecular basis of thrombin interaction with the γ′ peptide and broadens our understanding of how thrombin interacts with fibrinogen utilizing epitopes distinct from the active site. Exosite II and regions in close proximity to the Na^+ site offer the molecular scaffold for γ′ recognition by thrombin. Remarkably, these domains correspond to the epitope of thrombin that recognizes heparin [13], thereby explaining why the binding of the γ′ peptide and heparin are mutually exclusive [9,12] and why thrombin bound to fibrin is resistant to inactivation by antithrombin and heparin cofactor II [14,15].

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Fig. 1. (a) Surface rendering of thrombin (wheat), with residues of exosite II making contacts with the γ′ peptide highlighted in blue and orange. The γ′ peptide is shown as a CPK model (C in yellow) with its electron density in green, contoured at 0.8σ level. (b) Overlay of the structures of thrombin (surface in wheat) bound to the γ′ peptide (CPK model, C in yellow) and heparin (CPK model, C in green) [13]. The γ′ peptide and heparin overlap remarkably well and share the same epitope for binding to thrombin. Relevant thrombin residues are highlighted in blue and orange as in panel A. The thrombin mutant R77aA was expressed, purified, and tested for activity as described previously [17]. The γ′ peptide with Tyr_P residues, $V^{408}RPEHPAETEY_PDSLY_PPEDDL^{427}$ (MW 3375.5), was prepared as described earlier [7]. The phosphorylated γ′ peptide is functionally equivalent to the native sulfated form [9,12]. Crystals of the complex were obtained using the hanging drop vapor-diffusion method. Thrombin and γ′ peptide were mixed at 1:10 M ratio and incubated at 4 °C for 2 h. The protein complex was concentrated to 5 mg/ml in 40 mM choline chloride and 20 mM Tris, pH 7.5, and 2 μl of the protein complex was mixed with 2 μl of the crystallization buffer containing 25% PEG 4000 and 100 mM HEPES sodium salt buffer, pH 7.5 and left to equilibrate at 23 °C. Crystals were grown in about 2 weeks. The crystal was cryoprotected in 32% PEG 4000, 100 mM HEPES, pH 7.5 and 15% glycerol for 3 min and frozen in liquid nitrogen. X-ray data were collected on an ADSC Quantum-315 CCD detector of the BIOCARS Beamline 14BMC at the Advanced Photon Source, Argonne National Laboratories (Argonne, IL). Data processing including indexing, integration and scaling was performed using the HKL2000 package [18]. Results of data collection and refinement are summarized in Table 1. The crystal structure was solved by molecular replacement with MOLREP from the CCP4 package [19] using the coordinates of the PPACK-inhibited slow form of human thrombin (PDB entry 1SHH) [17] as the starting model. Refinement and electron density map generation were carried out using CNS [20] and 5% of the reflections were selected randomly and set aside as a test set for cross-validation [20]. Noncrystallographic symmetry (NCS) restraints were applied to the two protein molecules in the asymmetric unit during refinement except for N- and C-terminal regions of the light chain and the 99-loop of the heavy chain due to differences between the two molecules. Electron density maps showed that one molecule of γ′ peptide was bound to the surface area between the second molecule in the asymmetric unit and a symmetry-related molecule. Thereafter, the peptide was added to the model and the water molecules were added interactively at the end of each refinement cycle using Fo–Fc peaks over 3σ. In the final cycle of refinement, Na^+ was added to the model based on the electron density and coordination geometry. The coordinates of the structure of the thrombin mutant R77aA in complex with the γ′ peptide have been deposited to the Protein Data Bank (accession code 2HWL).

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